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Pork Meat as a Potential Source of *Salmonella enterica* subsp. *arizonae* Infection in Humans

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***Salmonella enterica* subsp. *arizonae* was isolated from 13 of 123 slaughtered pigs in central Greece. The samples cultured were feces, ileum tissue, mesenteric lymph nodes, and gallbladder swabs. A total of 74 isolates from 492 samples were identified as *Salmonella* spp. by use of standard laboratory culture media and two commercial micromethods and by use of a polyvalent slide agglutination test for the detection of O and H antigens. Among them were 19 (25.68%) suspected to be *S. enterica* subsp. *arizonae* according to analysis with standard laboratory culture media. Of those, 14 were identified as *S. enterica* subsp. *arizonae* by the API 20E (bioMérieux, France) and the Microgen GnA+B-ID (Microgen Bioproducts, Ltd., United Kingdom) identification systems. All the isolates were tested for resistance to 23 antimicrobials. Strains identified as *S. enterica* subsp. *arizonae* were resistant to 17 (70.8%) antibiotics. The highest proportions of resistance were observed for sulfamethoxazole-trimethoprim (71.4%), tetracycline (71.4%), ampicillin (64.3%), and amoxicillin (57.1%). Two isolates were resistant to aztreonam (7.1%) and tigecycline (7.1%), used only for the treatment of humans. Thus, pork meat may play a role in the transmission of antibiotic-resistant *S. enterica* subsp. *arizonae* to human consumers. This is the first report of *S. enterica* subsp. *arizonae* isolation from pigs.**

Pork is a major source of food-borne salmonellosis in the European Union and around the world (1). Therefore, the European Food Safety Authority (EFSA) (2) considers many *Salmonella* serovars isolated from pigs, among which are Choleraesuis, Enteritidis and Typhimurium, important for public health (3, 4). Although *Salmonella enterica* subsp. *arizonae* is typically associated with reptiles, sporadic cases of human infection, related mainly to children, have also been reported (5–7). In such cases, the source of the microorganism is thought to be rattlesnake meat and some other animal products, especially poultry, as well as pet turtles (7, 8). Pork meat, however, is not among them, perhaps because this subspecies appears, due to the lack of scientific reports, to be a pathogen that is not important in pigs. *S. enterica* subsp. *arizonae* became important to public health during the 1980s, when several cases of human infections were associated with widespread use of rattlesnake meat, capsules, and powders (5, 9). These rattlesnake products were used by the Latino communities of the southwestern United States as forms of alternative medicinal therapies (10). Also, although adult human cases of infection by this microorganism are rare and perhaps underreported, the microbe should be considered a risk factor for infants and immunocompromised individuals having a history of contact with reptiles (6) and perhaps consumers of pork meat that was undercooked or unsafely handled during the cooking process (11).

S. enterica subsp. *arizonae* was first described in 1939 and named *Salmonella dar es salaam*, after the African city where it was first isolated from diseased chuckwallas, horned lizards, and Gila monsters (12). Since then, the placement and nomenclature of this species was continuously debated until it was placed, regardless of its many atypical similarities with the genus *Salmonella*, into the genus *Arizona*, which has only one species, *A. hinshawii* (4). In later years, the development of DNA homology studies placed it back in the genus *Salmonella* and in the group of subspecies III (13–15). *Salmonella* subspecies III, later named *S. enterica* subsp. *arizonae*, has since been isolated from reptiles, fowl, turkeys, ducks, dogs, cats, monkeys, goats (10), and wild boars (16). To our knowledge, however, it has not been reportedly isolated from pigs.

We report here the isolation of *S. enterica* subsp. *arizonae* from the carcasses of finishing pigs in central Greece.

MATERIALS AND METHODS

Samples and sampling procedures. A total of 492 samples were collected from 123 randomly selected pigs during slaughtering between September 2012 and March 2013. From each pig, samples were collected from various sites and samples from relative tissues were pooled. Thus, 123 samples each from pooled feces, pooled ileum, mesenteric lymph nodes, and gallbladder swabs were examined, in amounts and with the methods recommended by the 2002 ISO *Salmonella* rule 6579 applied to food and animal feeding stuffs (17). These samples were collected from 15 swine finishing farms, representing 10% of the swine finishing farms in central Greece.

Laboratory examination of samples. (i) Isolation and serotyping of *Salmonella* spp. Samples were cultured by standard culture methods following the 2002 ISO *Salmonella* rule 6579 (17). Briefly, after enrichment in buffered peptone water (BPW) (CM104; Oxoid), 0.1 ml of culture was inoculated onto modified semisolid Rappaport-Vassiliadis medium (MSRV) (BK191HA; Biokar) and incubated at 41.5 ± 1°C for 24 ± 3 h. A loopful of microorganisms taken from the edge of the MSRV colony was

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TABLE 1 Proportions of resistant and sensitive *S. enterica* subsp. *arizonae* isolates

Antimicrobial agent	No. (%) of isolates that were:	
	Resistant	Susceptible
Amoxicillin	8 (57.1)	6 (42.9)
Amoxicillin-clavulanic acid	5 (35.7)	9 (64.3)
Ampicillin	9 (64.3)	5 (35.7)
Ampicillin-sulbactam	0 (0)	14 (100)
Aztreonam	1 (7.1)	13 (92.9)
Cefotaxime	0 (0)	14 (100)
Cefoxitin	5 (35.7)	9 (64.3)
Ceftazidime	1 (7.1)	13 (92.9)
Ceftiofur	0 (0)	14 (100)
Ceftriaxone	0 (0)	14 (100)
Cefuroxime	0 (0)	14 (100)
Chloramphenicol	5 (35.7)	9 (64.3)
Colistin	3 (21.4)	11 (78.6)
Doripenem	0 (0)	14 (100)
Enrofloxacin	2 (14.3)	12 (85.7)
Gentamicin	0 (0)	14 (100)
Kanamycin	1 (7.1)	13 (92.9)
Nalidixic acid	3 (21.4)	11 (78.6)
Penicillin G	14 (100)	0 (0)
Rifampin	14 (100)	0 (0)
Sulfamethoxazole-trimethoprim	10 (71.4)	4 (28.6)
Tetracycline	10 (71.4)	4 (28.6)
Tigecycline	1 (7.1)	13 (92.9)

TABLE 2 Antibiotic resistance profiles of *S. enterica* subsp. *arizonae* isolates recovered from pig samples

Isolate no.	Sample source	Phenotypic antibiotic resistance of isolate to[a]:
1	Feces	AML, AMP, FOX, CT, P, RD, SXT, TE
2	Gallbladder	AML, AMP, C, P, RD, SXT, TE
3	Gallbladder	AML, AMC, AMP, FOX, C, P, RD, SXT, TE
4	Ileum	CT, P, RD
5	Gallbladder	C, P, RD, SXT, TE
6	Feces	P, RD
7	Feces	P, RD
8	Gallbladder	AML, AMP, C, ENR, K, NA, P, RD, SXT, TE
9	Feces	AMC, AMP, P, RD
10	Ileum	AML, AMP, C, P, RD, SXT, TE
11	Feces	AML, AMC, AMP, FOX, ENR, NA, P, RD, SXT, TE
12	Gallbladder	P, RD, SXT, TE, TGC
13	Gallbladder	AML, AMC, AMP, FOX, P, RD, SXT, TE
14	Lymph nodes	AML, AMC, AMP, ATM, FOX, CAZ, CT, NA, P, RD, SXT, TE

[a] AML, amoxicillin; AMC, amoxicillin-clavulanic acid; AMP, ampicillin; ATM, aztreonam; FOX, cefoxitin; CAZ, ceftazidime; C, chloramphenicol; CT, colistin; ENR, enrofloxacin; K, kanamycin; NA, nalidixic acid; P, penicillin G; RD, rifampin; SXT, sulfamethoxazole-trimethoprim; TE, tetracycline; TGC, tigecycline.

inoculated onto xylose-lysine-deoxycholate agar (XLD) (CM469; Oxoid), brilliant green agar (BG) (CM329; Oxoid), and *Salmonella-Shigella* agar (SS) (1.07667; Merck), all selective for *Salmonella* spp. Suspect colonies were examined by the API 20E (bioMérieux, France) and the Microgen GnA+B-ID (Microgen Bioproducts, Ltd., United Kingdom) systems, suitable for Gram-negative bacteria, supplemented by the oxidase, indole, and urease tests, triple sugar iron agar, lysine iron agar, and citrate utilization.

Isolates identified as *S. enterica* subsp. *arizonae* were tested for the presence of O and H antigens using a polyvalent slide agglutination test (Remel Europe, Ltd.; Dartford, England).

(ii) Antimicrobial susceptibility testing. The antimicrobial susceptibility of isolates identified as *S. enterica* subsp. *arizonae* was determined for 23 antimicrobials according to the disk diffusion method using Mueller-Hinton agar (LMLAB 39). *Escherichia coli* ATTC 25922 was used as the quality control strain. Interpretation of results followed the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (18), the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (breakpoint tables for interpretation of MICs and zone diameters, version 3.1, 2013 [http://www.eucast.org]), and Galani et al., 2008 (19). Thus, for those antimicrobials for which breakpoints were not available, a strain was considered resistant when it showed an inhibitory zone below 12 mm (as do most of the organisms known as resistant) and as safely sensitive when having an inhibitory zone above 15 mm (as do most of the sensitive organisms on the lists). The antimicrobials used were selected according to their use for animal and human infections. They were amoxicillin (30 μg), amoxicillin-clavulanic acid (20/10 μg), ampicillin (10 μg), ampicillin-sulbactam (10/10 μg), aztreonam (30 μg), cefotaxime (30 μg), cefoxitin (30 μg), ceftazidime (30 μg), ceftiofur (30 μg), ceftriaxone (30 μg), cefuroxime (30 μg), chloramphenicol (30 μg), colistin (50 μg), doripenem (10 μg), enrofloxacin (5 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), penicillin G (10 μg), rifampin (30 μg), sulfamethoxazole-trimethoprim (23.75/1.25 μg), tetracycline (30 μg), and tigecycline (15 μg). Isolates exhibiting resistance to at least three antimicrobial agents belonging to different antimicrobial classes were considered multidrug resistant (MDR) strains (20).

RESULTS

Isolation and serotyping of *Salmonella* spp. The API 20E micromethod identified 14 out of 492 samples (2.8%), originating from 13 pigs, as positive to *S. enterica* subsp. *arizonae*. The same 14 isolates examined by the Microgen were identified as *S. enterica* subsp. *arizonae* (4 isolates), other *Salmonella* spp. (5), and different bacteria species (7). The strains identified as *S. enterica* subsp. *arizonae* by the API 20E were isolated from feces (6), ileum (3), mesenteric lymph nodes (2), and the gallbladder (7). All 14 isolates were found to be strongly positive by the slide agglutination test for the presence of *Salmonella* O and H antigens.

Antimicrobial susceptibility testing. Isolates examined showed varied resistance patterns (Tables 1 and 2). Twelve of 14 isolates were resistant to at least three antimicrobial categories, thus considered MDR, and all 14 were resistant to penicillin G and rifampin. From the remaining antimicrobials, the highest resistance rates were observed for sulfamethoxazole-trimethoprim (71.4%) and tetracycline (71.4%), followed by ampicillin (64.3%) and amoxicillin (57.1%). Low resistance rates were seen for aztreonam, ceftazidime, kanamycin, and tigecycline (7.1% each). All isolates were susceptible to ampicillin-sulbactam, cefotaxime, ceftiofur, ceftriaxone, cefuroxime, doripenem, and gentamicin.

DISCUSSION

The isolation of *S. enterica* subsp. *arizonae* from pig carcasses has, to our knowledge, never been reported previously. In the present study, regardless of the biochemical microsystem used, some pigs were identified as carriers of this subspecies. Thus, pork meat could be a possible source of *S. enterica* subsp. *arizonae* transmission to consumers.

Due to the rare reporting of the isolation of this subspecies from food-producing animals, molecular confirmation is needed for explaining observed variations in the utilization of nutrients

incorporated in different commercial biochemical micromethods. An example is lactose incorporated in the Microgen system as a separate test. All isolates were found lactose negative with this system, although 50% of them were slow lactose fermenters, as previously reported (6), when cultured on *Salmonella-Shigella* (SS) and MacConkey agars. This could be one of the reasons the Microgen identified only four (28.6%) isolates as *S. enterica* subsp. *arizonae*. Another is the fewer years that this method has been used compared to the API 20E and/or the use of the Microgen mainly for the placing of Gram-negative bacteria isolated from humans. Thus, for increasing the accuracy of its database, it needs, perhaps, enrichment with information from animal isolates. Such problems and the usual practice of discarding lactose-fermenting bacteria as nonpathogenic (21) could play a roles in the rarity of isolating *S. enterica* subsp. *arizonae*. The API 20E system, on the other hand, used for many decades in identifying microorganisms from humans and animals, identified 14 isolates as *S. enterica* subsp. *arizonae* with a very high probability (99.7%).

However, regardless of the proportions given by each phenotypic identification method used, *S. enterica* subsp. *arizonae* was isolated from slaughtered pigs, thus making them a probable source for human infection. The evident disagreements between the two micromethods used for first recognition of *S. enterica* subsp. *arizonae* point to the need to molecularly type them to clarify the source of disagreements. This requires an expense that is not available to all, especially under an economic crisis. Thus, for overcoming difficulties in the interpretation of the present results, lysine-iron agar, suggested many decades ago as a useful aid in identifying the *Arizona* group within the family of *Enterobacteriaceae* (22), was used. All 14 isolates were found positive to this test.

Regardless of difficulties encountered in the phenotypic placement of these Gram-negative isolates, the antimicrobial profiles of them are of clinical interest (Tables 1 and 2). Resistance to antimicrobials was high for those of low price, an observation indicative of a farmer's policy on the selection of antibiotics and the public health implications this may have. The economic crisis, forcing farmers to select cheaper antibiotics, could also further increase the resistance of microorganisms, such as *S. enterica* subsp. *arizonae*. Such increases could actually help this particular rare *Salmonella* subspecies increase its virulence, thus leading to its spreading among the pork industry and becoming a public health risk in the long run. The CLSI document M100-S21 (18) considers ampicillin a representative for the resistance patents of amoxicillin. However, they are low-cost agents, thus routinely used in Greece for prophylactic and therapeutic purposes. For this reason, a higher disk content for amoxicillin (30 μg) was selected for comparing the results and to derive information for practical use. Also interesting was the observed resistance to chloramphenicol. Chloramphenicol has been banned since 1994 by the European Union for use in food-producing animals (see the chloramphenicol summary report by the European Agency for the Evaluation of Medicinal Products [http://www.ema.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-_Report/2009/11/WC500012060.pdf]). Thus, the isolates found resistant were, perhaps, derived from human sources, an additional indication of an emerging risk factor for public health. This is supported also by the finding of two isolates resistant to aztreonam and tigecycline, agents used only in humans. There are two possible sources for these isolates, either animal care takers, because unofficial use of these antibiotics is impossible due to costs, or the transfer of resistance genes within the carrier animal between different species of microorganisms, including other *Salmonella* serovars (23). The latter could be molecularly investigated by comparing the resistance genes and resistance-conferring structures of related microorganisms from the same animals or farms.

Another finding of interest is resistance observed to ceftazidime, which is an expanded-spectrum cephalosporin. This drug is considered by the WHO as a critically important antimicrobial for human medicine (24), and one of the therapeutics of choice for the treatment of *Salmonella* infection, together with aztreonam (25). The one isolate resistant to aztreonam is indicative of strains transferred from humans to animals during handling. However, *Salmonella* spp. resistance to aztreonam is not a rare observation for humans and animals (26–31). A major factor in the development of antibiotic resistance, a risk to public health, is the simultaneous use of therapeutic agents, such as ampicillin, trimethoprim-sulfamethoxazole, expanded-spectrum cephalosporins, and fluoroquinolones, in humans and food-producing animals. The development of resistant bacteria is threatening the efficient treatment of human infections (see the Joint FAO/World Organisation for Animal Health [OIE]/WHO Expert Workshop on Non-Human Antimicrobial Usage and Antimicrobial Resistance scientific assessment [http://www.who.int/foodsafety/publications/micro/nov2003/en/]). Pigs have been recognized as the primary reservoir of multiresistant bacteria (32), showing increased virulence, thus increasing the costs of disease to the pig industry (1, 33) and becoming sources of such bacteria for humans.

Most cases of human salmonellosis are foodborne, and pork is frequently a source of *Salmonella* (3). Undercooked meat is one source, but also important are improper in-home food handling and preparation and inadequate hand washing or washing of utensils during preparation of other materials consumed raw, such as salads. Cross-contamination of food materials via contaminated surfaces from raw meat has been implicated in foodborne outbreaks (11). Thus, *S. enterica* subsp. *arizonae* could be an emerging foodborne pathogen in the future, originating from the consumption of pork meat and becoming difficult to treat if it becomes multiresistant to antibiotics used in human medicine.

In summary, the present results demonstrate that *S. enterica* subsp. *arizonae*, a subspecies mainly associated with cold-blooded animals, is also infecting pigs, possibly making pork meat a source for human infection. *S. arizonae* could, under selective pressure, adapt to a new host, such as the pig, increasing its importance as a risk factor for humans. Improvements in the methods of molecular typing of this subspecies could provide new insight regarding the relatedness of rare serovars, such as those of *S. enterica* subsp. *arizonae*, to animal and human infections.

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